and innovative fused capillary nanolitre flow cells in NMR probes, and further development in cryoprobe technology along with the use of improved processing procedures, will continue to enhance the sensitivity of HPLC-NMR coupling. As a microanalytical method, HPLC-NMR allows the detection of various groups of natural compounds and other biomolecules in the nanogram or even picogram range and, therefore, can contribute to the solution of problems of biochemical, physiological and chemoecological research.

See also: **II/Chromatography: Liquid:** Mechanisms: Reversed Phases; Nuclear Magnetic Resonance Detectors. **III/Medium-Pressure Liquid Chromatography. Natural Products:** Liquid Chromatography. **Terpenoids: Liquid Chromatography.**

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Supercritical Fluid Chromatography

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The mild elution temperatures and the wide range of molecular masses it can accommodate makes supercritical fluid chromatography (SFC) particularly applicable to natural products. It is becoming the preferred method for the separation of enantiomers, and is especially useful for combined or hyphenated techniques. It forms a link between liquid chromatography (LC) and chromatography (GC), it has capabilities between the two and shares the instrumental set-ups of both, so both capillary column and packed column applications are recorded here.

The advantages and disadvantages of the method are debated elsewhere, but some of its strong points are indicated here. In all, 99% of supercritical fluid applications use supercritical carbon dioxide, since it has the great advantage that it is a nontoxic, nonflammable, pure, cheap mobile phase that presents no disposal problems. The greatest usefulness of SFC comes in connection with supercritical fluid extraction, which has received much attention for the isolation of natural products. If a substance can be extracted from plant or animal material with a supercritical fluid and some of the extract can be diverted to an online SFC, the course of the extraction can be followed very easily. Many of the applications of SFC recorded for natural products are of this type. The

greatest disadvantage of supercritical carbon dioxide is its relatively nonpolar nature. In its solvent powers, it resembles hexane at lower pressures, becoming slightly more polar at higher pressure. The polarity of the fluid can be increased by the addition of a small proportion of a highly polar organic solvent, miscible with the supercritical CO_2. This is most commonly methanol. The solubility of water in supercritical CO_2 is too low to be of much use to increase the polarity (but see below). The proportion of the so-called modifier solvent can range from 1 to 25%, but the critical point of the mixed fluid increases with the proportion of organic liquid and the advantages mentioned in this paragraph are steadily eroded with increasing proportion of modifier. In the chromatography of natural products, therefore, SFC is most useful for products of low polarity, such as terpenes, lipids and essential oils.

Free fatty acids, methyl esters, mono-, di-, and triglycerides can all be separated by SFC methods. The technique is particularly useful for triglycerides. Because triglycerides lack a useful UV absorption for high performance liquid chromatography (HPLC) and are at the limit of volatility for GC, they are not easy to separate and quantify without conversion to methyl esters. Using SFC with capillary columns and a flame ionization detector, they can be analysed directly. For capillary column determination of free fatty acids in an ethanol extract of *Sabal serrulata* two alternatives have been proposed: derivatization of the carboxyl group and saturating the CO_2 with water. Both methods produce a drastic improvement in resolution. Hydroxy acids are too polar for direct determination, and the separation of triglycerides is improved by the addition of a little methanol or acetonitrile, which then is detrimental to the use of a flame detector. It is possible to convert free carboxylic acids to their methyl esters in a flow-through system with CO_2 containing 10 mol% methanol at 80°C over a cation exchange resin in the H form. Capillary columns with a flame ionization detector have been used to separate the fatty acids and alcohols from hydrolysis of jojoba oil. The unhydrolysed portion of the wax esters could also be seen in the chromatogram. Wool wax alcohols from hydrogenated lanolin have been examined similarly. Using a microextractor, the triglycerides of a single cotton seed kernel were extracted by supercritical fluid extraction (SFE) and linked directly to SFC for analysis. In a rare example of application to insects, the same microcell (at 45°C and 20.2 MPa or 200 atm) has been used to extract the cuticular hydrocarbons and waxes from the cuticle of a dried fruit beetle *Carcophilus hemipterus*, which were then separated by capillary SFC. The results, probably not optimized, were not as good as normally obtained by GC. Prostaglandins have been separated on a capillary column at 100°C with a CO_2 density gradient.

Staby and Mollerup have produced a comprehensive review of the separation and chromatography of fish oil constituents with supercritical fluids. These include triglycerides, free fatty acids, methyl and ethyl esters, cholesterol, α-tocopherol, phospholipids and squalene. The review is particularly directed towards pilot plant separations. Another review by Borch-Jensen and Mollerup in 1997 compared chromatographic systems for natural products like fats, seeds, oils and tissues.

The less polar steroids are usefully separated by SFC. Eleven steroids, including testosterone, oestrone, oestradiol, oestriol, cortisone and hydrocortisone, can be separated in less than 2 min with 6.1% methanol in CO_2 on a cyanopropyl HPLC column. Even bile acid conjugates (e.g. glycocholic acid and taurocholic acid) can be subjected to SFC. The ecdysteroids (insect moulting hormones, with a polyhydroxycholesterol structure) have been separated on packed columns under very similar conditions. Boronic ester derivatives of a diol functional group in some ecdysteroids improve selectivity for that group. The ecdysteroids, which have a strong UV chromophore, are also found in many plants of diverse type, and extracts of plants can be very quickly scanned for the presence of so-called phytoecdysteroids. With the coupling of a mass spectrometer to the SFC column, separation and identification of phytoecdysteroids can be performed in a matter of minutes.

Capillary SFC has been used to analyse the terpenes of some aromatic plants and the results compared with those obtainable by GC. Thyme (*Thymus vulgaris*) gave the same information as GC; for peppermint (*Mentha* × *piperita*) and basil (*Ocimum basilicum*) the separation is much better by GC but SFC quantification is more reliable. The monoterpenes of lemon peel oil have been examined on a packed SFC column. Using two different silica columns (Nucleosil 100 and Spherisorb Si) linked in series with SFC Fourier transform infrared (FTIR; see later) eight sesquiterpenes (longicyclene, longifolene, aromadendrene, ledene, valencene, *cis*- and *trans*-calamenene and humulene) have been separated and identified, as have five sesquiterpenes from copaiba balsam and ylang-ylang oil under similar conditions.

Carotenes, with their strong visible and UV chromospheres, are ideal subjects for SFC. In spite of their low polarity, there are frequent reports that addition of a very small amount of an organic modifier improves selectivity. Vitamin A can exist in five pairs of *cis-trans* isomers which can be separated at

temperatures below 50°C, so avoiding any fear of isomerization.

More Polar Compounds

As already indicated, many examples of SFC of natural products are found where extraction with a supercritical fluid (SFE) and chromatography are coupled. A classic SFC separation, performed in 1982, was the separation of caffeine, theophylline and theobromine with CO_2–methanol. The pungent phenolic oil of ginger (*Zingiber rhyzoma*) has been analysed for [6]-gingerol (see **structure**), [8]-gingerol and [10]-gingerol. SFE-SFC has been used in the extraction of ginkgolides (oxidized diterpenes from the leaves of *Ginkgo biloba*), paclitaxel (the antitumour agent Taxol) from *Taxus brevifolia*, and the antimalarial artemisinin and its precursor artemisinic acid from *Artemisia annua*. The chromatography of artemisinin was carried out on both a capillary column with CO_2 (and 3% methanol) and a flame detector and on an aminopropyl packed column with a CO_2–methanol gradient (17.18 MPa and 40°C) and an evaporative light-scattering detector. The packed column method was faster: both compounds were eluted in 7 min as against 25 min by the capillary method. Paclitaxel has a strong UV absorption, but the ginkgolides do not, so the evaporative light-scattering detector is very helpful. Ginkgolide A has a molecular formula $C_{20}H_{24}O_9$, ginkgolide B $C_{20}H_{24}O_{10}$, paclitaxel $C_{47}H_{51}NO_{14}$, and artemisinin $C_{15}H_{22}O_5$, which shows that highly functionalized compounds can be suitable for SFC.

Limonoids from hexane extracts of the plants *Aphanamixis polystacha*, *Harpephyllum caffrum* and *Entandrophragma delevoyii* have been analysed by capillary SFC. A liquid crystal-modified polysiloxane phase that discriminated molecular shape was used in a capillary column to separate triterpene acids, including geometric isomers, from *Disoxylum pettigrewianum*. The natural insecticide azadirachtin (see **structure**) is usually analysed by reversed-phase HPLC, but because the extract obtained from Neem seeds usually contains a lot of less polar contaminants, including triglyceride oil, the column has to be flushed with pure methanol or acetonitrile at the end of each run, and total cycling time can be of the order of 1 h. With packed column SFC, using an aminopropyl silica or cyanopropyl silica column and CO_2–methanol (24 : 1, v/v) mobile phase at 20.6 MPa (3000 psi or 207 bar) at 55°C and at a flow rate of 2 mL min^{-1}, the oily impurities are eluted in the solvent front, good resolution and good peak shape are obtained, and the cycle time is about 20 min (**Figure 1**). The example illustrates how SFC in the right situation can have marked advantages over other chromatographic methods.

The cannabinoids and their metabolic products have been examined on capillary columns. SFC is perhaps not the most useful way to examine alkaloids, but a number of separations have been made. Six opium alkaloids (narcotine, papaverine, thebaine, ethylmorphine, codeine and morphine) have been separated from poppy straw on an aminopropyl column with CO_2–methanol–methylamine–water. Seven ergot alkaloids were separated from *Claviceps purpurea* and eight pyrrolizidine alkaloids from *Senecio anonymus*.

Flavonoids from citrus fruits that contain several methoxy groups (hexa- and heptamethoxyflavone, tangeretin, nobeletin, sinensetin and others) were separated on a silica column with CO_2–methanol.

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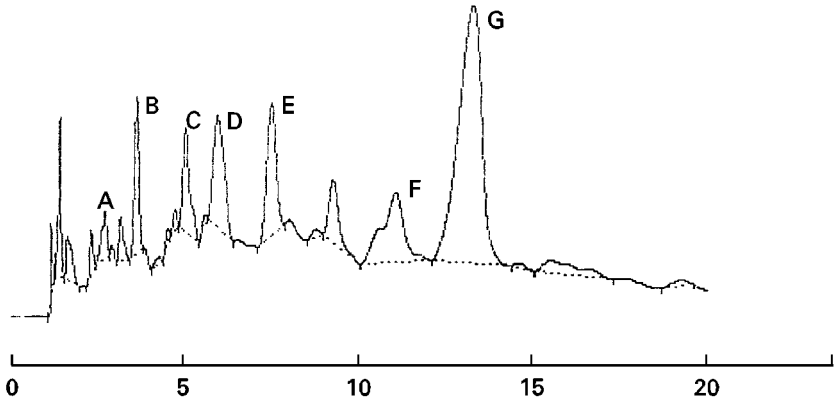


Figure 1 SFC chromatogram of an extract of triterpenoids from the seeds of *Azadirachta indica*, on a Spherisorb cyanopropyl column (150 × 4.6 mm i.d.) of 5 μm particle size, flow rate 2 cm^3 min^{-1} of CO_2–methanol (94 : 6) at 3000 psi (20.7 MPa) and 50°C with UV detection at 217 nm. Compounds are: A, nimbin; B, salannin; C, 6-desacetylsalannin; D, 3-desacetylnimbin; E, 3-tigloylazadirachtol; F, 3-acetyl-1-tigloylazadirachtinin; G, azadirachtin. Unpublished results of A.P. Jarvis and E.D. Morgan.

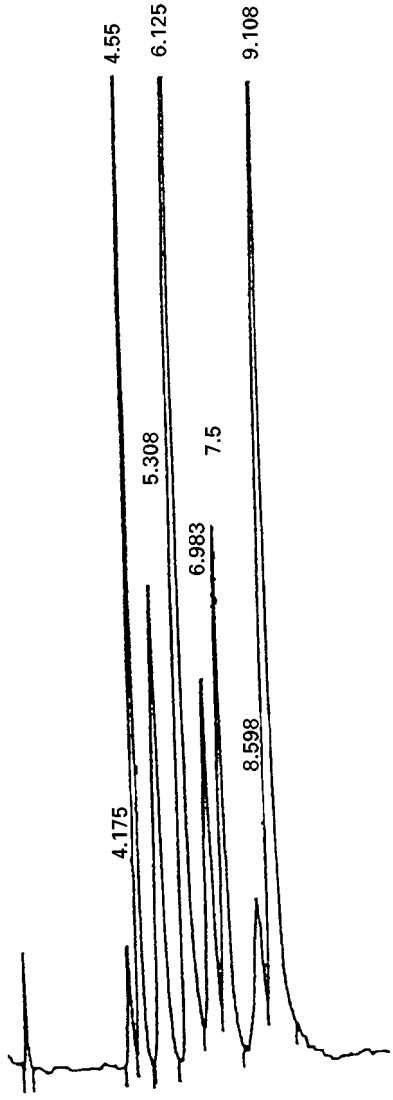


Figure 2 SFC of monosaccharides on Zorbax TMS column (250 × 4.6 mm i.d.), flow rate 5 cm^3 min^{-1} of CO_2–modifier (80 : 20, v/v) at 200 bar (20 MPa) and 60°C. The modifier was methanol–water–triethylamine (91.5 : 8.0 : 0.5, v/v). Compounds in order of elution are D-ribose, m-erythrose, D-xylose, xylitol, L-sorbose, D-mannose, D-glucose and mannitol. (Reproduced with permission from Salvador A, Herbreteau B, Lafoose M and Dreux M (1997) Subcritical fluid chromatography of monosaccharides and polyols using silica and trimethylsilyl columns. *Journal of Chromatography A* 785: 195.)

A number of monosaccharides, polyols and glycolipids have been separated on silica and trimethylsilyl-bonded silica under subcritical conditions (41°C, 200 bar, and CO_2–methanol 80 : 20, or a modifier of methanol with 4% or 8% water; **Figure 2**).

An extensive review (in French) by Lubke cites 207 references on the advantages of SFC for the analysis of natural products.

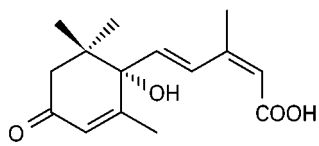
At the time of writing there is increasing interest in the use of subcritical water at 100–200°C as a mobile phase with divinylbenzene polymer columns for the chromatography of highly polar compounds like alcohols, phenols, amino acids and flavones. Clearly any compounds to be analysed must be stable to hydrolysis, as must the stationary phase. As temperature and pressure increase, water becomes less polar – at 200°C it is rather nonpolar – so by controlling these conditions a fluid phase of intermediate polarity similar to the water–methanol mixtures used in HPLC can be achieved.

Chiral Separations

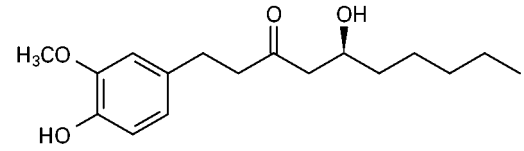
Another advantage of SFC is in the separation of enantiomers. Chiral separations can be carried out at lower temperatures (often with greater efficiency than by GC on the same phases), and on larger molecules than with GC and with improved resolution over HPLC. The subject is still at a very early stage of development, with new discriminator phases appearing rapidly. If insufficient resolution is obtained under supercritical conditions, some investigators recommend subambient (and consequently subcritical) conditions. Separations have included the plant hormone abscisic acid (see **structure**), benzoin, ephedrine, mandelic acid, tropic acid (**Figure 3**), underivatized amino acids and their derivatives. For the separations shown in Figure 3, the composition of the mobile phase had the largest effect on retention, peak shape and enantioselectivity, with temperature the second most important influence. Tyrosine and tryptophane enantiomers are separated under subcritical conditions on a Chirobiotic T phase at 30°C and 200 bar isocratically with 40% modifier (methanol–water–glycerol, 92.8 : 7.0 : 0.2 v/v) in CO_2 with 0.1% triethylamine and 0.1% trifluoroacetic acid. Enantioseparations of five lignans were carried out on polyWhelk-O between 0°C and − 42°C with 5–15% methanol in CO_2 with α values from 1.28 to 1.44. The potential of chiral separations of less volatile pheromones unsuitable for GC has not yet been explored.

Hyphenated Methods

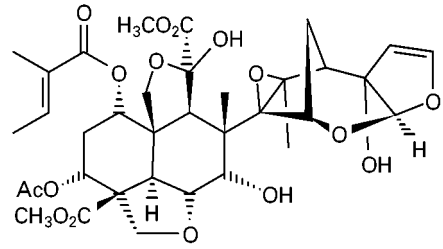
All the predictable couplings of SFC to spectroscopic methods have been tried. SFC-FTIR has been used to look for isomerized fatty acids in the triglycerides of partially hydrogenated soya bean oil and in the free fatty acids after hydrolysis using a flow-through FTIR cell. SFC is more easily coupled to a mass spectrometer than HPLC, therefore many examples of SFC-mass spectrometry (MS) can be



Abscisic acid



[6]-Gingerol



Azadirachtin

found. The spectra resemble chemical ionization spectra, with prominent M+ or M + 1 ions. Alkaloids of *Securidaca longipendunculata* have been separated by SFC-MS and SFC-MS-MS with a moving belt. Thermospray in the electron ionization mode with an SFC gradient was used for the indole alkaloids of *Catharanthus roseus* to identify 60 compounds. By SFC-UV 10 major alkaloids and 30–40 minor compounds were detected in the extract. A few antibiotics, including penicillin, cyclosporin, tetracyclin, oxytetracyclin and mitomycin C have been determined, in some cases after extraction from blood. Ecdysteroid spectra can be varied by the operating conditions to give additional ions for M-H_2O, M-2H_2O, etc.

There are a number of examples of direct coupling between SFC and proton nuclear magnetic resonance (NMR) spectrometers. CO_2 has the great advantage of being transparent and only a small proportion of CD_3OD may be required. For polar compounds, subcritical D_2O can be used (see discussion above); it is both transparent and does not generate a very high pressure. The vapour pressure of water at 200°C is only 1.55 MPa (225 psi or 15.5 bar). Resolution of

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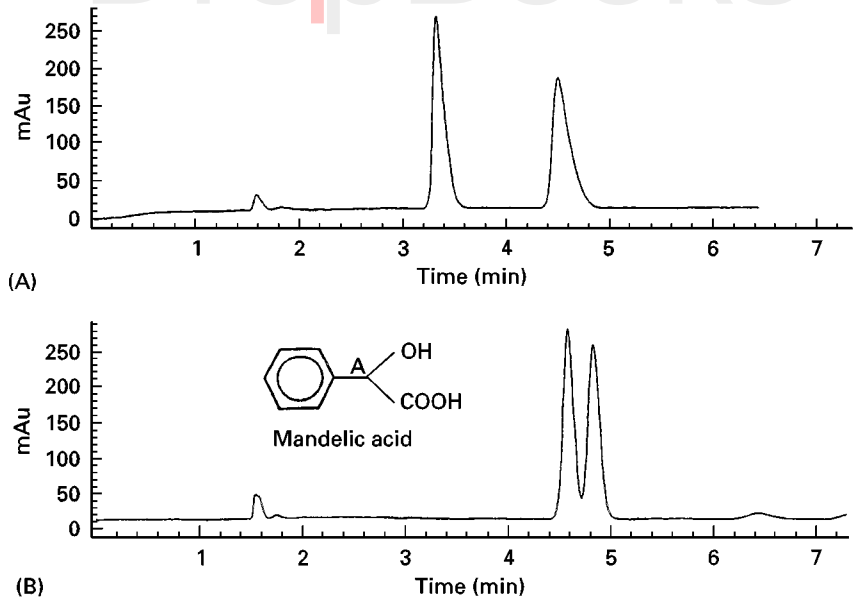

Figure 3 Separation of the enantiomers of mandelic acid on (A) Chiralpak OD (3,5-dimethylphenylcarbamate derivative of cellulose coated on 10 μm silica gel) and (B) Chiralpak AD (3,5-dimethylphenylcarbamate derivative of amylose coated on 10 μm silica gel). Both columns were 250 × 4.6 mm i.d., at flow rate 2 $cm^3\ min^{-1}$ of CO_2–methanol containing 0.1% triethylamine and 0.1% trifluoroacetic acid and programmed from 5% modifier (5 min) to 30% at 5% min^{-1} at 200 bar (20 MPa) and 30°C. (Reproduced with permission from Medvedovici A, Sandra P, Toribio L and David F (1997) Chiral packed column subcritical fluid chromatography on polysaccharide and macrocyclic antibiotic chiral stationary phases. *Journal of Chromatography A* 785: 159.)

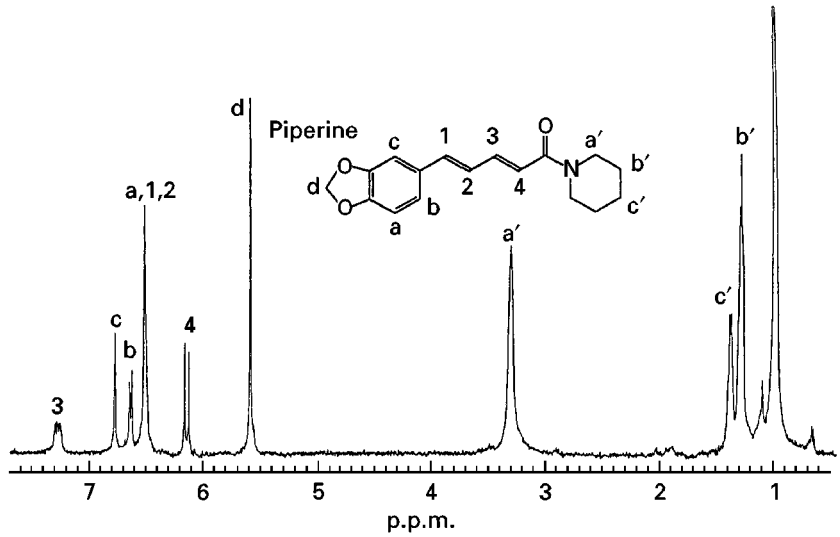

Figure 4 A stopped-flow ^{1}H NMR spectrum at 400 MHz of piperine extracted from pepper with supercritical CO_2 at 0.5 cm^3 min^{-1}. Pressure was 294 bar (29.4 MPa), and temperature 44°C. (Reproduced with permission from Albert, 1997.)

the NMR spectra under continuous flow approaches the quality of conventional spectra. SFC-NMR has been applied to vitamins and a range of natural products, including extracts of coffee, hops and pepper (**Figure 4**). Two problems are, firstly, the dependence of NMR signals on pressure, and secondly, the increased spin-lattice relaxation times in a supercritical fluid.

Mycotoxins from *Fusarium roseum* culture extracts have been studied with a combination of SFC-UV and SFC-MS. Some experiments have been conducted with SFE-SFC-NMR-MS and we can expect to see more of such techniques and the hyphens extended.

Future Directions

SFC will not replace GC or HPLC, but predictions are dangerous. We already see new ideas coming forward, such as subcritical cryoseparations and use of superheated water as the mobile phase, that will extend its potential. In the field of polymers, surface active compounds are being used to render growing polymers soluble in supercritical CO_2. The advantages of SFC are such that we can expect every opportunity to be seized to extend its possibilities, particularly in the areas of chiral separations and hyphenated methods.

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